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We determined whether female deer ticks Ixodes dammini (Acari: Ixodidae) can be inseminated repeatedly and whether sperm from either mating take precedence in fertilizing eggs. By infesting deer with irradiated male I. dammini, the abundance of these vector ticks may effectively be reduced.

The spirochetal agent of Lyme disease, Borrelia burgdorferi, disseminated from the guts of infected Ixodes dammini ticks following host attachment and appeared in slaiva before rapid engorgement commenced. This salivary route of infection appears to be the route of Lyme disease transmission in nature.

We compared the development of the Lyme disease spirochete, Borrelia burgdorferi, in subadult rabbit-feeding Ixodes dentatus with that in mouse-feeding I. dammini. Because I. dentatus

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feeds solely on rabbits, and these hosts may be extraordinarily abundant in nature, this tick provides potential for a hidden enzootic cycle of natural Lyme disease transmission.
Taken together this penultimate series of observations on the dymanics of transmission of tick-borne pathogens indicates that (1) vector ticks may be eliminated by sterile-made release, (2) spirochetes are delivered via the saliva of ticks, and (3) that spirochetal pathogens of rodents may be transmitted in the same manner as those of mice.

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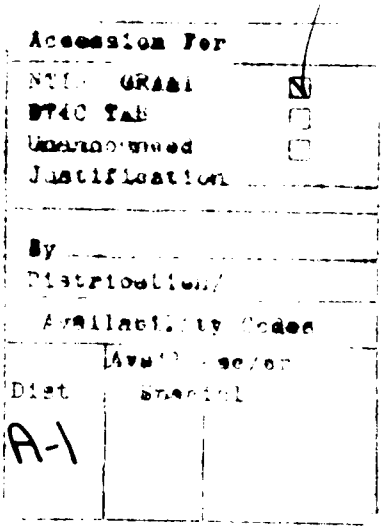

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SUMMARY

This report describes progress on research in which the main goal was to define the physiological properties of ticks that determine the infectivity of pathogens that they transmit.

We determined whether female deer ticks Ixodes dammini Spielman, Clifford, Piesman & Corwin (Acari: Ixodidae) can be inseminated repeatedly and whether sperm from either mating take precedence in fertilizing eggs. Such information is essential to the design of attempts to reduce the fertility of these vectors of Lyme disease. Although spermatophores are present in about half of questing female ticks, they are present in virtually all those found on deer; the abundance of males on deer exceeds that of females and copulation is common. Females must be inseminated before commencing the rapid engorgement phase of feeding. Males need not be in attendance during feeding, provided that the female has been inseminated preprandially. Thus, preprandial insemination suffices to stimulate rapid engorgement, but less blood is taken than when the female is preprandially inseminated. Either insemination effectively fertilizes eggs. The fertility of eggs from females, sequentially inseminated by irradiated and by nonirradiated males, mainly reflected the last insemination. Cobalt-irradiated males mate effectively, and their sperm compete with those of nonirradiated males. Sperm from the last of 2 sequential inseminations fertilize most of the eggs. By infesting deer with such irradiated male I. dammini, the abundance of these vector ticks may effectively be reduced.

The spirochetal agent of Lyme disease, Borrelia burgdorferi, disseminated from the guts of infected Ixodes dammini ticks following host attachment and appeared in saliva before rapid engorgement commenced. In about half of the adult ticks studied, spirochetes disseminated to the hemocoel at 4 days after attachment. Of these, half produced saliva containing spirochetes. Infected nymphs correspondingly produced spirochete-containing saliva at 3 days postattachment, the time at which saliva was first collected. We conclude that contact between spirochete-infected ticks and a host provides the stimulus that ultimately results in dissemination of infection and delivery via saliva. This salivary route of infection appears to be the route of Lyme disease transmission in nature.

We compared the development of the Lyme disease spirochete, Borrelia burgdorferi, in subadult rabbit-feeding Ixodes dentatus with that in mouse-feeding I. dammini. Rabbits were infected with spirochetes by the bites of I. dammini that had been infected naturally in a zoonotic site

in which rabbits were scarce. Larval ticks of both species were permitted to engorge simultaneously on each of these infected hosts. Spirochetes were present in the guts of about half of the resulting nymphal I. dentatus and most of the I. dammini that developed. An experimentally infected nymphal I. dentatus, in turn, infected a rabbit. Because rabbits are the only mammals that are attacked by I. dentatus, and these hosts may be extraordinarily abundant in nature, this tick provides potential for a hidden enzootic cycle of natural Lyme disease transmission

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

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Andrew Spielman 14 Nov 91

PI - Signature Date

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Presence of Lyme Disease Spirochetes in Ticks Feeding on Sparsely Infected Hosts

Larval deer ticks (Ixodes dammini) regularly become infected by the Lyme disease spirochete (Borrelia burgdorferi) during their prolonged periods of attachment to reservoir rodents (Donahue et al. 1987). Although spirochetemia has been reported (Bosler et al. 1983. Burgdorfer and Gage 1987), experimentally infected hamsters and naturally infected white-footed mice (Peromyscus leucopus) appear virtually aspirochetemic (nonpublished). Indeed, our attempts to demonstrate spirochetal infection in rodents indicate that "xenodiagnosis" is far more effective than is direct examination or culture of blood, and this suggests that the microorganism may tend to be concentrated by the feeding tick. That procedure, however, requires at least a month of incubation and depends upon the ability of the spirochete to proliferate and develop in the tick. In the event that Lyme disease spirochetes accumulate at the site of attachment and are ingested by the feeding tick, rapid diagnosis of active infection might be facilitated by immediately examining the gut contents of feeding ticks. Accordingly, we determined whether spirochetes become evident in or near ticks during the course of feeding. In particular, we recorded the presence of Lyme disease spirochetes in the guts of previously noninfected larval ticks that had fed on spirochete-infected rodents for varying periods of time and in the skin on which these ticks were feeding.

A. Materials and Methods

Deer ticks were reared from female adults collected in the fall of 1987 in Ipswich, Massachussetts (USA). Females were permitted to feed on the ears of New Zealand white rabbits and to oviposit at 25°C and 95 % RH. Resulting larvae were maintained under these conditions for at least one month and thereafter at 5°C.

White-footed mice, P. leucopus, were derived from an established laboratory colony derived from animals captured in the same Ipswich site. These rodents serve as the natural hosts for the subadult stages of the tick and the main reservoir for the Lyme disease spirochete. In addition, month-old female ICR mice and golden Syrian hamsters (Johnson et al. 1988) were used for in these experiments. A Massachusetts strain of the Lyme disease spirochete was obtained from field-collected ticks and maintained in white-footed mice by serial passage through subadult deer ticks. Each of these experimental hosts was infected via the bites of infected nymphal ticks and

examined for spirochetal infection by xenodiagonsis after about one month.

To detect spirochetes in ticks, they were removed from hosts by gentle traction. The gut was then dissected from each tick into a small quantity of phosphate-buffered saline (PBS) on a glass slide, smashed beneath a cover slip and examined by fluorescent microscopy using a direct fluorescent antibody technique employing a fluorescein-isothiocyanate-conjugated polyclonal antibody raised in rabbits (Steere et al. 1983).

Skin samples (1-2mm across) were taken from the ears of nembutal-anesthetized golden Syrian hamsters at a point just beneath the tick attachment site. Samples from the back were taken by punch-biopsy and were 4mm in diameter. Each sample was individually teased in a small quantity of PBS on a microscope slide using forceps and with the aid of a dissecting microscope. The preparation was then smashed with another glass slide and observed by the direct fluorescent antibody method.

B. Results

First, we determined whether spirochetes could be recognized in the gut contents of feeding ticks and when they could most readily be seen. Thus, noninfected larvae were placed on the ears of anesthetized hosts. Spirochetes could not be seen before the third day of attachment in any animal tested (Table 1). Some infections were evident on that day, and all but a few ticks contained spirochetes on the fourth day. Spirochetal infection similarly approached unity in ticks that had become replete and fallen from their hosts after the fourth day of feeding. The number of spirochetes ingested from infected hosts increased in parallel with prevalence of infection, regardless of species of host. White-footed mice, ICR mice and hamsters appeared to provide similar numbers of spirochetes. We concluded that ticks ingested numerous spirochetes mainly after 3 days of attachment and during the final phase of feeding.

In addition, we determined whether spirochetes may concentrate in skin near the point of insertion of the mouthparts of a feeding tick. Thus, skin was biopsied from hamsters at the time that ticks were sampled (Table 1). Spirochetes could readily be seen in samples of skin taken on the fourth day of attachment, but not in those taken earlier.

C. Discussion

These experiments demonstrate that feeding ticks may be used to rapidly and dependably detect the Lyme disease spirochete in infected rodent hosts. Indeed, xenodiagnosis

has been employed in this manner (Donahue et al., 1987; Levine et al., 1985; Piesman et al., 1987) and with similar efficacy. But, this procedure requires many weeks of incubation, assumes that the larval tick survives through the larval-nymphal molt and that spirochetal infection is retained until after nymphal ecdysis. At present, clinical practice is hampered by an inability to detect active Lyme disease in human or animal hosts. Serological evidence of infection, for example, fails to present convincing evidence of treatment failure. It may be that feeding ticks may similarly be used to detect spirochetes in medical or veterinary medicine.

The presence of numerous spirochetes near the point of attachment of engorging ticks suggests that Lyme disease spirochetes may accumulate in skin. Such organisms seem readily demonstrable in sectioned skin, particularly when sampled from the advancing edge of the erythema migrans lesion (Kornblatt et al., 1984; Aberer et al, 1988; Park at al., 1986; Berger et al., 1983; Berger, B.W., 1984; De Koning J. and Hoogkamp-Korstanje J.A.A., 1986). Our observations suggest that the spirochete may reside in the skin throughout infection and that vector ticks may imbibe these organisms from fluids in that tissue. We failed to detect spirochetes by culturing the blood of several of the rodents in these experiments (nonpublished), and other attempts to demonstrate spirochetemia have generally failed (Anderson et al., 1987). It may be that the spirochete resides in the skin and accumulates near the feeding tick due to some taxis operating late in blood feeding. It is interesting that white mice support spirochetal infection. Such well-characterized laboratory animals may prove useful in other studies on Lyme disease.

Sperm Precedence in an Ixodes Tick

Female ixodid ticks take only one blood meal, from which their entire reproductive output is derived. Mating and blood feeding generally are closely linked, as illustrated by the female's inability to feed unless males are in attendance. The spermatophore conveys the essential signal that induces rapid engorgement (Pappas and Oliver (1972), a relationship that applies generally to metastriate ticks, and possibly to Ixodes species (prostriates), as well (Randolph, 1980; Diehl et al., 1982; Oliver, 1986). Sexual behavior of Ixodes ticks differs naturally from that of all other ixodid ticks primarily in that many seem to be capable of mating off as well as on the host. Other ticks invariably mate during or immediately before blood feeding. In nature, I. ricinus (L.) (Graf, 1978; Gray, 1987) and I. persulcatus (P. Sch.) (Babenko et al., 1979) generally become inseminated before the host is encountered. Indeed, female I. muris (Bishopp and Smith) invariably mate before feeding, and their non-feeding mates do not seek hosts

(Smith, 1944). Such preprandial mating provides opportunity for Ixodes females to become inseminated repeatedly and for sperm competition to occur (Parker 1970), a condition that would not apply to other ixodid ticks in which mating occurs preprandially.

The recent holarctic emergence of Lyme disease as a severe public health burden places research priority on approaches that may lead toward strategies for reducing the abundance of the vector Ixodes dammini (Spielman, Clifford, Piesman & Corwin) and other members of the I. ricinus group of species. Although little is known of the mating behavior of these ticks, such techniques as sterile male release, the use of chemosterilants and possibly even satirizing males and hybrid sterility (Ribeiro and Spielman, 1986; Sutherst, 1987) may hold promise for protecting people against tick-borne infection. Information on sperm precedence, existing for some argasids (Sternberg et al., 1973) but lacking for any ixodid, would provide an essential conceptual basis for the development of such techniques. In order to prepare a physiological basis for an attempt to reduce the fertility of female I. dammini, we examined ticks inseminated both in nature and the laboratory. We determined whether these ticks frequently become inseminated in nature before attaining contact with a host and whether such preprandial insemination affects the ability of a female to engorge and ultimately to produce fertile eggs. In particular, in doubly inseminated females, we determined whether sperm from one or another of these inseminations fertilized a disproportionate number of eggs.

A. Materials and Methods

a. Field observations

Ticks were collected on Great and Nantucket Islands, near Cape Cod, Ma., and along Argilla Rd. in Ipswich, Ma. Questing adult ticks were collected by flagging vegetation. Other adults were removed from deer taken by licensed hunters at the Crane Reservation, in Ipswich. Immediately upon collection, all specimens were segregated as to sex in separate vials. Abundance of males and females was recorded, as was the frequency with which females were paired with attendant males in copula. A sample of females from each collecting event were dissected in saline, the uterus removed and inspected under X400 magnifications in order to detect spermatophores.

b. Laboratory experiments.

Virgin adults were obtained by placing laboratory reared blood-fed nymphs in individual Eppendorf tubes. On eclosion, females and males were cloistered in 10 ml. vials,

and kept at 25° and 99% rh. For the fertilization experiments males were irradiated with 10 krads of gamma radiation from a Cobalt 60 irradiator at a rate of 26.1 cGy/sec. This was judged to be a sterilizing dose (e.g. Pappas & Oliver 1972), yet did not otherwise affect the ticks behavior. Ticks were induced to mate off the host by placing 25-30 virgin males and females in a moist vial for at least 14 days. Mating invariably was observed, and insemination was verified by dissecting 2-3 females from each vial. Ticks were permitted to engorge by feeding on the ears of laboratory rabbits. They were confined to the ear enclosed by an elastic stocking taped to its base. Females were weighed to within 0.0001g before and after feeding. Following drop-off, females were placed in individual vials and monitored for oviposition and subsequent hatching of the eggs. Hatching was scored as the percent of eggs producing larvae, as described by Gladney & Drummond (1971).

B. Results

In a preliminary series of observations, we determined how frequently female I. dammini are inseminated in nature before attaining contact with a host. These ticks were collected by flagging in the various study sites at intervals between the months of September and May. They were segregated as to sex immediately upon capture and their uteri excised and examined for the presence of spermatophores. For comparison, feeding ticks were removed from deer killed at the Crane Reservation, in Ipswich. Between 30 and 72% of females flagged from vegetation were inseminated (Table 2). Although females seemed to have been inseminated more frequently as their season of activity progressed, this trend did not prove to be significant. Females taken from deer were more frequently inseminated than were those collected by flagging ($Z=-7.2$, $P>>0.001$). In addition, copulating ticks were found more frequently on deer (63.3 7.9%) than on vegetation (4.2 1.2%). Males were more abundant on deer than were females (63.3 5.2%), as compared to their representation in samples of questing ticks (47.7 7.7%). Thus, although female I. dammini generally mate while feeding on hosts, nearly half are inseminated before attaining contact with these animals.

We then compared the effects of pre- and per-prandial insemination on the ability of these ticks to feed on blood and to reproduce. In the first series of experiments, 6-week old virgin laboratory-reared females were permitted to feed on the ears of rabbits for as long as 10 days; others co-fed with males; and others were confined with males for 2 weeks before being placed without males on the ears of rabbits. Although virgin females imbibed some material from these rabbits (doubling their pre-prandial 17.01.0 mg

weight), none entered the rapid engorgement phase of feeding; nor did they produce eggs (Table 3). Although about as many pre-prandially inseminated females engorged as did those that were per-prandially inseminated, pre-prandially inseminated females did not engorge as fully ($T=7.57$, $P=<0.001$). Eggs were similarly fertile, however, regardless of time of insemination. In the second series of experiments, field-collected specimens were flagged from Great Island during November and held without males for a month before being placed, either alone or with males, on the ears of rabbits. They weighed 19.01.0 mg before feeding. Although only about half of those not co-feeding with males entered the rapid engorgement phase of feeding, virtually all females engorged when feeding in the presence of males. In addition, such per-prandially inseminated females fed more fully than did those feeding alone ($T=4.04$; $P<<<0.001$). About half of these field-collected females appear to have mated in the field, and this corresponds to the results of direct observation of spermatophores (Table 2). Here too, virtually all deposited eggs were fertile. Thus, these ticks must be inseminated before commencing the rapid engorgement phase of feeding; pre-prandial insemination serves to stimulate rapid engorgement, but not as much as does per-prandial insemination; and either insemination effectively fertilizes eggs.

The order of precedence of sperm in doubly inseminated females was then investigated. In order to determine whether irradiated males became effectively sterilized, we permitted virgin females to co-feed on a rabbit either with non-irradiated or with irradiated males. Both groups of females engorged successfully, but none of the eggs of those mated to irradiated males hatched (Table 4). Non-feeding females were then confined with irradiated males and others with non-irradiated males. After 2 weeks, females that had been confined with irradiated males were placed on the ears of rabbits with non-irradiated males, and vice versa. Order of insemination did not affect degree of engorgement (Table 4). Females of both groups deposited egg masses proportional in size to the volume of blood ingested. Fertility, however, depended mainly upon the nature of the second insemination. When nonirradiated males were present on the host, eggs generally were fertile. Conversely, when irradiated males were on the host, less than 15% of eggs were fertile. This establishes that sperm from the last insemination fertilize most, but not all, of the eggs.

In addition, we evaluated the ability of irradiated males to compete for mates. Similar numbers of laboratory-reared virgin females (25) were placed on each ear of a rabbit together with 20 irradiated and 20 normal virgin males. All of the 39 females that engorged oviposited. About half of these egg batches (19) were completely infertile (Table 4). Of the fertile egg batches, all but 3

hatched normally. Thus, the course of radiation that was employed does not impair the ability of treated males to compete for mates in the short term.

C. Discussion

The appearance of many inseminated females in the questing samples indicates that mating off the host is a common event in I. dammini. This result coincides with the observations of Graf (1978) and Gray (1987) on I. ricinus and those of Babenko et al. (1979) on I. persulcatus. The degree of insemination, varied from sample to sample and particularly from site to site, reflecting, perhaps, differences in tick densities between these sites. Interestingly, questing females collected on Great Island, where tick densities had recently been reduced by deer removal (Wilson et al. 1988), were somewhat less frequently inseminated than were those collected elsewhere. Immature I. dammini feed chiefly on white-footed mice Peromyscus leucopus and to a lesser extent on white-tailed deer Odocoileus virginianus. But adults, for reasons unknown, feed mainly on deer, and never on mice (Spielman et al. 1985). The diurnal drop off rhythm of larvae and nymphs feeding on mice coincides with periods of host inactivity, thus concentrating them in the nest of the host (Mather & Spielman 1986). It is possible that contact between emerging males and females is facilitated by these rhythms, and that mating occurs in the nest before the adults disperse in search of their final host. It may be that females that were non-inseminated when flagged from vegetation may have fed as nymphs on deer or on some diurnal host as a bird, a chipmunk or a vole. We find that female I. dammini may mate twice and that sperm from the second insemination assume a degree of precedence in fertilization. Such precedence is common in some polygamous insects (e.g. Etman & Hooper 1979; Waage 1984; Simmons 1987), although the reverse is true in spiders such as Frontinella pyramitela where the sperm from the first insemination fertilizes most eggs (Austad 1982). Why sperm competition ends one way or another is hardly a random matter. It is reasonable to interpret the sexual behavior of any organism as the interim result of a continuous conflict between the sexes, each attempting to optimize reproductive success while minimizing parental investment. Furthermore, the particular ecological circumstances under which these behaviors evolve will constrain and to a large extent shape, the behavior in question. Male I. dammini would benefit by inseminating females before they themselves seek hosts; they would benefit most if the inseminated female attaches to a host and fails to mate again. This female can engorge (although not as fully as when per-prandially mated) and eventually lay fertile eggs. A lesser benefit would accrue to the first male if this female were to mate again while feeding. Fewer than 15% of her eggs would be fertilized by sperm from

the first mating event. We argue that males may limit their pre-prandial matings because sperm derived from such matings are less likely to fertilize eggs than are sperm from per-prandial matings and because males are capable of inseminating only a limited number of females (Gladney and Drummond, 1971; Thompson et al., 1980). Pre-prandial insemination benefits females by insuring them against the possibility that they may fail to encounter a mate on deer. Because female ticks must find a host in order to reproduce, the precedence enjoyed by per-prandial matings would represent an "evolutionary reward" for males that find hosts effectively. The mechanics of sperm precedence remain obscure because ticks appear not to possess structures particularly adapted for removing sperm such as those borne by certain male insects (Thornhill & Alcock 1983). We have observed copulating males with their mouthparts inserted in a female's genital aperture throughout the period of feeding. Such prolonged mating contact may serve a dual function. Opportunity might be provided for males to ingest or otherwise remove previously deposited sperm before depositing their own. Once they inseminate the females themselves, the continued physical presence of the male would deny other males opportunity to mate.

The competitive ability of irradiated male I. dammini and the relative precedence of per-prandial insemination encourages us to suggest that the force of transmission of Lyme disease may be reduced by infesting deer with irradiated males. Mass rearing of this tick, however, is extremely laborious. Nevertheless, in sites where transmission of Lyme disease is intense, a public health program combining deer reduction with repeated release of irradiated laboratory-reared males near deer feeding stations might provide an environmentally acceptable brake to the presently increasing pattern of transmission of Lyme disease.

Fine structural evidence for the penetration of the Lyme disease spirochete Borrelia burgdorferi through the gut and salivary tissues of Ixodes dammini

Lyme disease is a systemic inflammatory human illness identified in its early stages by a characteristic expanding annular skin lesion, erythema chronicum migrans, and causing in later stages various neurologic, cardiac, and arthritic disorders (Steere and Malawista, 1979; Reik et al., 1979; Steere et al., 1980). This multisymptomatic disorder results from the bite of an ixodid tick, Ixodes ricinus in Europe and I. dammini and I. pacificus on the ease and west coasts of the U.S.A., respectively (Anderson et al, 1983; Lane and Burgdorfer 1987). Lyme disease is growing in epidemic proportions, becoming the tick-borne disease of greatest public health interest in recent times (Piesman 1987).

Larval I. dammini ingest the spirochetal agent of Lyme disease, Borrelia burgdorferi (Burgdorfer et al., 1982), while feeding on an infected reservoir host such as the white-footed mouse Peromyscus leucopus (Piesman and Spielman, 1979; Mather et al., 1987), and the bacterium can later be found in the midgut of the unfed nymph in close association with the brush border of the midgut epithelium (Burgdorfer et al., 1982; Benach et al. 1987). Transovarial transmission of B. burgdorferi from female to larvae is less than 1% in I. dammini (Piesman et al. 1986); however, I. pacificus appears to inherit the infection more frequently (Lane and Burgdorfer 1987). Ixodes dammini parasitizes a wide range of birds and mammals (Magnarelli et al. 1984; Anderson et al. 1986) and all stages have been observed to feed on humans (Main et al. 1981); however, human disease generally results from the bite of the nymph (Steere et al. 1978).

The route followed by the spirochete as it passes from vector tick to reservoir host has been the subject of controversy. Infected I. dammini stimulated by pilocarpine produce spirochete-laden saliva (Ribeiro et al. 1987), thus providing experimental evidence for saliva as the vehicle of transmission. Morphological studies failed to demonstrate the spirochete in salivary tissues (Benach et al. 1987), supporting the belief that the spirochete may be transmitted by regurgitation while the tick is feeding (Burgdorfer 1984).

For salivary transmission to occur, the spirochetes must pass from the gut to the salivary glands. There has been no evidence of spirochete invasion into either the midgut epithelium or the salivary glands, although small numbers have been seen in the hemolymph (Benach et al. 1987; Ribeiro et al. 1987). The spirochetes may just "pass through" these tissues and this transient passage may make them difficult to find (Benach et al. 1987).

In the event of such transient spirochetal passage through the salivary tissues of feeding ticks, attempts to demonstrate salivary infection must span the period of nymphal attachment to the host. We employed a detailed electron microscopic study in an attempt to solve this controversial problem of transmission. We sought to determine whether B. burgdorferi invades the salivary acini and ducts. In addition we examined other tissues of the vector to determine the route and mode of migration of these zoonotic pathogens.

A. Materials and methods

Ticks

Three different groups of Ixodes dammini ticks were used in this study. Group 1 were nymphs derived from the eggs of an adult female collected on Great Island, MA in 1986. The hatched larvae were fed on a Borrelia burgdorferi infected, laboratory-bred white-footed mouse (Peromyscus leucopus). The Borrelia burgdorferi infection was derived from an isolate from Naushon Island, MA. Larvae were maintained at room temperature under conditions of saturated humidity from repletion through the molt to the nymphal stage. Nymphs at 25 weeks postlarval feeding (plf) were allowed to feed on an uninfected hamster for 48 h, removed, and thereafter processed for transmission electron microscopy (TEM). Unfed nymphs from this same group at 35 weeks plf were also processed for TEM.

Group 2 were nymphs derived from a female collected on Nantucket Island, MA in 1987. Larvae were fed on a B. burgdorferi infected wild P. leucopus from Nantucket Island, and they were maintained at room temperature under conditions of saturated humidity from repletion through the molt. In an attempt to catch the dissemination of the spirochetes from the gut of the nymph in response to feeding, three batches of nymphs at 37 weeks plf were allowed to feed on an uninfected hamster for 4-5, 12, or 24 h, and thereafter processed for TEM. Unfed nymphs from this same group at 45 weeks plf were also prepared for TEM.

The third group consisted of four wild adult female I. dammini collected in 1980 from vegetation in an endemic site in Massachusetts. The were fed on an uninfected rabbit for 5 days after which time they were removed and 24-48 h later they were precessed for TEM.

Detection of spirochetes

Light microscopy

Darkfield (DF) and direct fluorescent antibody (DFA) microscopy were used to detect spirochetes in the ticks. The gut and salivary glands were dissected in a drop of PBS on a microscope slide, covered with a cover slip, squashed, and examined for spirochetes under DF illumination at 640x magnification. After DF examination, cover slips were removed and slides were air-dried, fixed in acetone, and stored at -20^{o}. For DFA examination the slides were incubated with fluorescein isothiocyanate conjugated antibody as described by Steere et al. (1983). Antibodies were produced in rabbits immunized with cultured B. burgdorferi (courtesy of A.C. Steere, Yale University, School of Medicine).

Transmission electron microscopy

Ticks were dissected in either 0.85% NaCl (w/v) or directly in fixative composed of 2% glutaraldehyde (v/v) in 0.1 M cacodylate buffer, pH 7.2-7.4, containing 2% sucrose (w/v). Salivary glands, gut, and ticks halved longitudinally were immersed in two changes of ice-cold fixative for 1-2 h, followed by three washes in ice-cold buffer containing 4% sucrose (w/v). The tissue remained overnight in cold buffer and was postfixed the following day in cold 1% OsO4 (w/v) in 0.1 M cacodylate buffer, pH 7.2-7.4. Following postfixation the tissue was washed twice in cold distilled water, soaked for 1-2 h in ice-cold 0.5% (w/v) uranyl acetate solution, washed twice in cold distilled water, dehydrated in ascending alcohols (70%, 95%, 100%) at room temperature, and subsequently passed through two changes of propylene oxide. The tissue was then left uncovered overnight in a mixture of equal parts of propylene oxide and Epon and embedded in fresh Epon the following day. The Epon was polymerized at 60° for 2 days and cured for an additional day at 90°. Thick sections were cut with glass knives and stained with 1% (w/v) toluidine blue in 1% (w/v) sodium borate. Thin sections were prepared with a diamond knife, collected on copper grids, stained with uranyl acetate ad lead citrate, and viewed with a Phillips 300 transmission electron microscope.

B. Results

Ultrastructure of Borrelia burgdorferi in Ixodes dammini.

DF and DFA microscopy preceded our EM study and demonstrated the presence of B. burgdorferi in the guts of the nymphs. EM revealed the ultrastructure of the spirochete and its location in the tick tissues.

The spirochetes in the tick measure 0.19-0.25 um in diameter (Figs. 1,2) and are approximately 6 um in length (Fig. 3). An outer cell membrane or envelope surrounds the Borrelial cell (Figs. 1,2). Occasionally an amorphous layer is seen in some preparations loosely attached to the surface of the outer envelope (Figs. 10,14,17). Beneath the outer envelope in the elongated body of the cell, the protoplasmic cylinder, surrounded by the plasma membrane and external to this is the peptidoglycan-containing cell wall (Hovind-Hougen 1976; Klaviter and Johnson 1979). Below the plasma membrane is an electron-dense granular cytoplasm (Figs. 1,2,3,). An abundance of ribosomes lying mainly near the periphery of the protoplasmic cylinder contributes to the granular nature of the cytoplasm. The clearer areas nearer to the center of the protoplasmic cylinder (Fig. 2) contain the nuclear material (Hovind-Hougen 1976). The outer envelope is loosely associated with the underlying protoplasmic cylinder forming the periplasmic space, which

bulges out towards one side, creating a lateral ridge (Figs. 1,2). Within this ridge in the periplasmic space are found 8 to 14 periplasmic flagella, tubular in shape and approximately 13 nm in diameter. Obvious twists seen along the length of the spirochete body in longitudinal section (fig. 3) indicate its helical shape, the characteristic shape of all spirochetes. The periplasmic flagella are seen running along the helix of the cell.

Borrelia burgdorferi in organs and tissues of fed I. dammini nymphs

Borrelia in the gut

Nymphs from both groups 1 and 2 were fed on uninfected hamsters. During feeding the gut lumen enlarges as the ticks engorge themselves in the blood meal and B. burgdorferi can be seen suspended in the gut contents both in the central part of the lumen (Fig. 1) and along the periphery close to the microvillar brush border (Fig. 4). Fewer spirochetes appeared in the gut lumens of group 2 nymphs than in those of group 1.

In group 1 nymphs, which had fed for 48 h, a peritrophic membrane is present and spirochetes are found on both sides of this structure, in the endoperitrophic space mixing with the incoming blood meal (Fig. 1) as well as in the ectoperitrophic space adjacent to the microvilli (Fig. 4). No peritrophic membrane is present in the gut of group 2 nymphs which had fed for only up to 24 h (Rudzinska et al. 1982).

Borrelia invade the apical surface of the gut epithelium between the microvilli (Fig. 4); the apical plasma membrane seems to invaginate around the spirochetes. Intracellular spirochetes located in the apical epithelial cytoplasm are surrounded by a second outer membrane presumably derived from the invaginated host membrane, and the surrounding epithelial cytoplasm appears undamaged (Fig. 5). Spirochetes are found intercellularly in the gut sandwiched between adjacent cell membranes, apparently able to force the membranes apart as they move through (Figs. 6,7). Intercellular Borrelia do not possess the second outer membrane seen in the intra-cellular forms (Fig. 5). Borrelia are found in both groups of nymphs along the basal cell membrane and the basal lamina with the spirochetes pressed up against the latter; the basal plasma membrane yields to their presence and molds itself around them (Fig. 8). The majority of spirochetes in the gut epithelium were found in this location.

Borrelia penetrate the basal lamina of the gut into the homocoel(Fig. 9) and can also be found within the connective tissue surrounding the musculature of the gut.

Borrelia burgdorferi in the salivary glands

In group 1 nymphs fed for 48 h, Borrelia can be seen in the hemocoel in association with both type I and glandular salivary acini as well as with the salivary duct tissue (Figs. 10-16). The amorphous outer coat of these spirochetes is in direct contact with the basal lamina of the glands and ducts (Fig. 10).

Within the type I acini, Borrelia are found extracellulary in the spaces created by the deep basal infolding of the plasma membranes of the acinar cells. The spirochetes maintain their size and shape in these relatively large extracellular spaces (Fig. 11).

The cells of the glandular salivary acini are in close contact with each other only near the luminal surface. Most of the lateral and basal cell surfaces are exposed to rather large extracellular channels and Borrelia are seen within these spaces. Apically within the acinus, Borrelia are squeezed between adjacent cells (Fig. 12) and the parasites are somewhat compressed, at times, in these intercellular spaces.

After 48 h feeding, some of the cells of the glandular acini develop an extensive basal to apical cytoplasmic canal system formed by the invagination of the basolateral plasma membranes. These cells are similar to the f-cells described by Fawcett et al. (1981) in the type II glandular acinus of Rhipicephalus appendiculatus. Spirochetes in the large extracellular spaces formed by this convoluted membrane system appear undistorted in size and shape (Fig. 13). Borrelia are able to push up against the luminal plasma membranes of these cells causing them to evaginate into the lumen with the appearance of the bacteria trying to break out from the cell (Fig. 13). Free spirochetes in the lumen of these acini (Fig. 14) have the same morphology as those in the lumen of the gut (Fig.1).

Spirochetes are present also within the tissue of salivary duct cells (Fig. 15) as well as in the lumens of these ducts (Fig. 16).

Borrelia are also found in the salivary glands of each batch of fed group 2 nymphs predominantly between the basal plasma membrane of the salivary acini and the surrounding basal lamina with occasional spirochetes observed intercellularly within the acini and apically near the lumen. Fewer spirochetes are seen in the salivary glands of group 2 nymphs than in those of group 1.

Borrelia burgdorferi in other tissues

Borrelia are found in the hemocoel in association with nephrocytes, the amorphous coat of the spirochetes being in contact with the basal lamina of the nephrocytes (Fig. 17). No spirochetes have so far been seen within a nephrocyte; however, an extensive search has not been performed.

Spirochetes are also seen in association with Malphigian tubules, tracheal cells, and nervous tissue. Figure 15 illustrates the presence of a spirochete within a ganglion located adjacent to a salivary duct. Borrelia are also found occasionally in the brain of the nymph (Fig.18).

Borrelia burgdorferi in unfed nymphs of I. dammini

The gut lumen of the group 1 (35 week plf) unfed nymph is constricted with the microvilli interdigitating in the center. The lumen is devoid of most of the particulate matter that had been ingested with the larval blood meal, except for the Borrelia which had been ingested during the larval feeding and are now crammed into the spaces between the microvilli. After an extensive search no spirochetes could be found in the gut epithelium, the salivary glands, or any other tissue in these unfed nymphs.

As in the unfed group 1 nymphs, Borrelia are present in the gut lumen of the unfed group 2 nymphs (45 weeks plf) in close association with the microvilli (Fig. 19). In addition, there are spirochetes within the gut epithelium sandwiched between adjacent cell membranes (Fig. 20) as well as extracellularly between the basal plasma membrane and the basal lamina and within the external gut musculature (Fig. 21).

Borrelia are also found in type I and glandular salivary acini of these unfed group 2 nymphs (Fig. 22) as well as in the duct tissue primarily basally between the basal plasma membrane and basal lamina and occasionally intercellularly within the acinus. Spirochetes are also seen in nervous tissue in these unfed nymphs.

Unusual forms of B. burgdorferi

A number of strange forms having appearances similar to spirochetes are present in each experimental group. By far, the largest numbers of these unusual forms are found in the unfed group 2 45 week plf nymphs.

An interesting form having the appearance of three to five healthy-looking protoplasmic cylinders surrounded by intact plasma membranes all enclosed within a single outer envelope is found in the lumen of the gut of unfed group 1 nymphs (Fig. 23). Some of the protoplasmic cylinders are associated with periplasmic flagella and others within the same outer envelope are not. The form in Fig. 23 measures

approximately 0.39 X 0.53 um and is located near a deteriorating, slightly larger than normal parasite measuring approximately 0.28 um in diameter.

Another form seen in unfed group 2 nymphs is that of large, swollen, deteriorating parasites. These forms are observed in the gut lumen, gut epithelium,. surrounding gut musculature, and at the basal surfaces of the salivary acini (Fig. 24). The outer envelope of each form remains intact as the parasites increase in size up to 1.5 um. The protoplasmic cylinders likewise increase in size while becoming less electron dense and vacuolated. In some cases fragments of flagella can be seen in the periplasmic space (Fig. 24). Similar forms to these are seen also in fed group 1 nymphs.

Borrelia burgdorferi in adult I. dammini

Spirochetes are found in the gut of ticks that fed on an uninfected rabbit for 5 days (Figs. 2,3,25,26). The spirochetes measure 0.21-0.23 um in diameter and have 8 to 13 periplamsic flagella. A well defined peritrophic membrane is present, and spirochetes are found on both sides of this membrane. Borrelia can also be seen in the process of crossing the peritrophic membrane from the endo- to the ecto-peritrophic space (Fig. 26). In the ectoperitrophic space Borrelia are in close contact with the microvilli and are seen penetrating the apical epithelial surface (Fig. 25). The host plasma membrane seems to invaginate as the spirochetes approach and intracellular spirochetes near the apical surface are surrounded by a second outer membrane presumably derived from the host plasma membrane (Fig. 25).

C. Discussion

Our EM findings of B. burgdorferi within the lumens and ducts of the salivary glands in I. dammini nymphs establishes a salivary route for the transmission of the causative agent of Lyme disease. This important finding demonstrates that the spirochete can be transmitted via the saliva as early as 48 h after the start of feeding (and even earlier in systemically infected nymphs) and supports the work of Ribeiro at al. (1987), who found spirochetes in the saliva of pilocarpine stimulated nymphs after 3 days of feeding.

Borrelia burgdorferi enter the gut of I. dammini while the larva is feeding on an infected host. Unlike relapsing fever Borrelia, which disseminate shortly after feeding (Burgdorfer 1951; Diab and Solimann 1977), B. burgdorferi remain within the lumen of the gut.

At the start of the larval feeding, due to the lytic action of the tick's saliva, the ingested blood meal

consists mainly of lysed cellular material (Raikhel 1983). The first intact red blood cells can be found in the gut of feeding I. dammini larvae 10 h after attachment to a host (Rudzinska et al. 1984). Babesia microti, an intraerythrocytic parasite taken in with the blood meal at this time, is able to survive and metamorphose (Rudzinska et al. 1984). The digestion of blood in ticks occurs within the epithelium of the gut (Akov 1982), and the low amount of digestive enzymes present in the gut lumen may account for the survival of the parasites. By 27 h after larval attachment, a peritrophic membrane, a sac-like structure surrounding the incoming blood meal, forms in the gut. It acts as a barrier preventing the passage even of such small particles as ribosomes. Babesia microti are able to penetrate this membrane and invade the epithelium by means of a specialized arrowhead organelle (Rudzinska et al. 1982).

Similarly, the first intact B. burgdorferi probably appear in the gut of the feeding I. dammini larvae at about 10 h after attachment. By 27 h the peritrophic membrane forms in the larvae and may prevent the spirochetes from reaching and penetrating the epithelium. Unlike the Babesia which deteriorate in the gut lumen of the larvae and can no longer be found by 13-14 days after repletion (Rudzinska et al. 1984), B. burgdorferi are able to survive in the gut after repletion and through the molt to the nymphal state. Why the spirochetes remain in the gut lumen transtadially without penetrating the epithelium even after the peritrophic membrane has broken down is not known. With the onset of feeding in the nymph, these Borrelia are then stimulated to invade the gut epithelium and can be found in the hemolymph (Benach et al. 1987; Robeiro et al. 1987). It is not yet known whether it is the blood meal or simply the contact with the host that stimulates this dissemination (Ribeiro et al. 1987). Elevated temperature may play a role in this activity (Feng and Chung 1938; Ribeiro et al. 1987).

Once the nymph begins to feed and before the peritrophic membrane forms, the spirochetes are in contact with the gut epithelial cells and might be free to penetrate them. In insects the peritrophic membrane is secreted as a liquid by the epithelial cells of the gut (Richards and Richards 1977) and in many cases, parasites leave the gut lumen before the peritrophic membrane solidifies (Stohler 1961; Lawrence 1966). Once solidified, the peritrophic membrane acts as a barrier to the spirochete, as evidenced by the greater number of spirochetes that can be found in the endoperitrophic space (Fig. 26) than in the ectoperitrophic space and the gut epithelium. Some spirochetes may be able to pass through the peritrophic membrane or may be caught in it as it solidifies (Fig. 26).

Borrelia burgdorferi travel intercellularly through the gut epithelium. They are able to push adjacent cell membranes apart as they make their way from the lumen to the hemocoel (Figs. 6.7). Benach et al. (1987) suggest that the spirochetes enter the intercellular spaces of the gut epithelium during the process of breakdown and proliferation of the epithelial cells. We have, however, observed Borrelia penetrating the intact apical surface of this epithelium (Fig. 4). The intracellular forms were surrounded by a second membrane, presumably derived from the luminal plasma membrane (Fig.5). It might be that the spirochetes sometimes enter this way, but they prefer and eventually find the intercellular route as the great majority of spirochetes were found intercellularly.

Spirochetes have been found both inter- and intra-cellularly in different tissues of both invertebrates (Tyson 1970, 1974, 1975) and vertebrates (Miller and Wilson 1962). Intracellular forms were located both free and within cytoplasmic vesicles (Sykes and Miller 1971). Borrelia duttonii have been reported inter- and intra-cellularly in the coxal glands, nerve, and tracheal tissue of the tick Ornithodoros moubata (Aeschlimann et al. 1968). The intracellular forms were located free in areas of degenerating cytoplasm. There is no evidence that B. burgdorferi prefer the intracellular environment and they do no appear to cause any tissue damage as they pass through the gut epithelium or other tissues of I. dammini.

Once through the epithelium the spirochetes find themselves in the extracellular space between the basal plasma membrane of the gut epithelium and the basal lamina. Outside of the gut lumen the majority of spirochetes were found between the basal lamina and the basal plasma membrane in both the gut epithelium and salivary acini (Figs. 8,22). Borrelia burgdorferi seem to be able to move along the basal lamina of these organs with the basal plasma membrane yielding to their presence. The spirochetes are able to penetrate the basal lamina of the gut and enter the hemocoel (Fig. 9).

From the hemocoel, B. burgdorferi enter the salivary glands. The spirochetes' amorphous outer coat may play a role in tissue recognition (Cox 1983). It seems that adhesion and (or) penetration of the spirochete takes place when contact is established between the amorphous coat and the basal lamina of the tick tissues. Some investigators (Keh. et al. 1986) suggest that the outer coat of parasites may help them to avoid host defenses.

Unlike Ba. microti that invade only the glandular salivary acini, B. burgdorferi seem to have no such preference as they were found in both type I and glandular acini. The spirochetes easily pass through the

intercellular spaces of the salivary acini and are able to pass through even the closely opposed cell membranes along the luminal surface of the glandular acini. During feeding the cells of the glandular acini proliferate and secrete their granules; some then degenerate and some metamorphaose to take on an osmoregulatory function (Fawcett et al. 1981). It is conceivable that during these cellular events the spirochetes may readily pass through into the lumen of the acinus. In addition, spirochetes may be able to force their way through the luminal plasma membrane into the lumen of the acinus (Fig. 13). From here they can be easily transported into the salivary ducts along with the saliva. Borrelia burgdorferi may also enter the ducts directly from the hemocoel (Fig. 15).

Burgdorfer (1951) demonstrated attractions of B. duttonii towards specific tissues of O. moubata, specifically the salivary and coxal glands, reproductive organs, and nervous tissue. Spirochete migration towards these tissues appears not to be a random process. Indeed, the greatest concentration of spirochetes found outside of the gut lumen in the nymphs in our study was found in the salivary glands. Spirochetes may readily invade reproductive tissues in adult ticks, but our observations on nymphs precluded such and analysis.

Our fine structural evidence for the timing of spirochete migration from the gut is ambiguous. Evidence derived from group 1 is consistent with the previously reported observation that B. burgdorferi are generally confined to the lumen of the midgut of I. dammini until a day or so after infected nymphs attach to rabbits or hamsters (Ribeiro et al 1987). Spirochetes were already disseminated, however, in group 2 nymphs, even before attachment. Smaller numbers of Borrelia were seen in the gut of unfed nymphs of group 2 than in the gut of unfed nymphs of group 1 perhaps because group 2 spirochetes were already disseminated before feeding began. The lesser number of spirochetes in the lumen of group 2 nymphs might be the reason why we observed no apical epithelial penetration.

Thus spirochetes appear to be distributed in unfed Ixodes ticks in two divergent patterns. Perhaps the disseminated condition results when the infection is inherited. Although transovarial transmission in I. dammini is less than 1% (Piesman 1987), this may be one explanation since both groups were each derived from a single female. Comparable experimental batches from group 2 were at least 10 weeks older than those of group 1. Perhaps with increased age, group 2 nymphs developed a systemic infection. We plan to follow group 1 as it ages, to see if this might be so. The geographic origins of both groups

also differ, and this might attribute to the difference in dissemination.

In our study we found no difference between the morphology of spirochetes seen in the tick tissues and that described for cultured organisms (Holt 1978; Burgdorfer et al. 1982). The size of B. burgdorferi as measured here in the nymphs (0.19-0.25 um in diameter) is consistent with the 0.18-0.25 um description given by Burgdorfer et al. (1982).

Borrelial flagella are inserted subterminally and bipolarly and run along the length of the cell body where they overlap at the midpoint. Because of this overlap, sections taken near the midpoint have twice as many flagella as those taken more terminally. These 13-nm diameter flagella are typical of the unsheathed flagella found in Borrelia isolated from I. ricinus and I. dammini (Hovind-Hougen 1984). Negative-stained preparations of cultured spirochetes have yielded the most accurate determinations of flagellum number. Borrelia burgdorferi has 7 to 11 flagella, the number varying even within a single strain (Hovind-Hougen 1984). Borrelia with seven flagella have been the only forms isolated from American patients with Lyme disease (Hovind-Hougen et al. 1986). Our variable counts of 8 to 14 flagella may be attributed to differences in the location of the cut, the larger counts derived from sections taken nearer to the midpoint of the organism.

The loose association of the outer envelope with the protoplasmic cylinder may facilitate the formation of the unusual forms of Borrelia that we observed. The large swollen forms have been seen in spirochetes in aged cultures (Aristowsky and Hoeltzer 1924). Similar forms can be produced by placing spirochetes in hypotonic solutions (Umemoto and Manikawa 1980) and by exposure to antibiotics (Furudawa 1975; Barbour et al. 1982). The decrease in density of these forms, their general unhealthy appearance, and the large numbers found in unfed group 2 nymphs (Our oldest batch) suggest that these are simply damaged or deteriorating forms responding to deleterious conditions in the host or in culture. The forms containing what appear to be multiple protoplasmic cylinders were suspected to be reproductive forms (De Lamater et al. 196=50, 1951; Pillot et al. 1964; Pillot and Ryter 1865). Current evidence, however, points to transverse fission as the only means of spirochete reproduction (Barbour and Hayes 1986); the function of these forms remains a mystery.

In summary, the ultrastructural localization of Borrelia burgdorferi in Ixodes dammini lends important insight into the biology of Lyme disease. Without causing any damage to the tissue, the spirochetes are able to pass both inter- and intra-cellularly throughout the epithelium of the gut from the lumen to the hemocoel and from there to

the salivary glands and the salivary ducts. Showing no preference for acinar type, B. burgdorferi invade both the type I and glandular salivary acini as well as the salivary duct tissue. The finding of B. Burgdorferi in the lumens of the salivary acini and most importantly in the salivary ducts in our EM study, as well as in the saliva of the tick (Ribeiro et al. 1987), provides strong evidence for the salivary transmission of the Lyme disease spirochete. The antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of the tick (Ribeiro et al. 1985) make the saliva not only an efficient means of transport for the spirochete from vector to host but may also help to establish a favorable environment in the host facilitating spirochetal invasion.

Competence of a rabbit-feeding Ixodes tick as a vector of the Lyme disease spirochete

The Lyme disease spirochete, Borrelia burgdorferi appears to be maintained in eastern North America mainly in white-footed mice (Peromyscus leucopus). These rodents readily acquire infection and remain infectious for Ixodes dammini, the tick that perpetuates this infection in nature, for extended periods of time (Donohue et al 1987). Where the agent is enzootic, the vector appears to acquire infection mainly from mice, and infection in these hosts may virtually be universal (Levine et al 1985). These rodents are the most abundant mammals in such sites and serve as host to most subadult I. dammini (Piesman et al 1979). Deer, on the other hand, appear to play no role as reservoir hosts for this agent (Telford et al 1988). In certain enzootic island locations, where the fauna is depauperate, rabbits are the sole mammals that appear to be abundant enough to warrant consideration as alternative reservoir hosts.

Cottontail rabbits (Sylvilagus floridanus), which locally may be abundant in eastern North America where Lyme disease is intensely transmitted, occasionally are infested by potentially infected nymphal I. dammini (Piesman and Spielman 1979), and rabbits can experimentally be infected by the agent of this infection (Burgdorfer 1984). The narrow host-specificity of the ticks that most frequently parasitize these animals, I. dentatus and Haemaphysalis leporispalustris (Smith 1941), provides potential for a lagomorph cycle of transmission. Although the vector competence of such rabbit-specific ticks for B. burgdorferi has not been described, the demonstrated vectors mainly are members of the genus Ixodes (Burgdorfer 1984; Burgdorfer et al 1985; Burgdorfer and Gage 1987; Donahue et al 1987), thereby focusing attention on I. dentatus. It may be that rabbit-ticks maintain an alternative cycle of transmission of the Lyme disease spirochete. As a first step in assessing this possibility, we compared features of the

competence of I. dentatus, as a vector of the Lyme disease spirochete, with that of I. dammini, the main local vector. Transstadial survival of the spirochete was evaluated using an isolate of B. burgdorferi derived from a site in which rabbits were rare. In addition, we sought to transmit infection between rabbits via the bites of experimentally infected I. dentatus.

A. Materials and Methods

Larval I. dammini, used in these experiments, were derived from adult ticks swept from vegetation on Great Island, in West Yarmouth, Massachusetts that had engorged on noninfected laboratory rabbits, as previously described (Piesman et al., 1986). Rabbit-feeding ticks were obtained from Nantucket Island (Massachusetts), where these hosts were abundant. Thus, larval I. dentatus were obtained from gravid adult ticks that had been removed from cottontail rabbits shot on the grounds of the University of Massachusetts Nantucket Field Station. All ticks were held in vials containing a charcoal/plaster of paris base and maintained at 22^{o}C under a 16h photophase.

Our experimental isolate of B. burgdorferi was derived from adult I. dammini collected during October of 1986 on Great Island. About 40% of such ticks contain spirochetes (Piesman et al. 1986 and nonpublished). Infection was passed into female New Zealand white rabbits, Oryctolagus cuniculus, (Millbrook Farms, Wilmington, MA), each weighing 2kg, via the bites of 20-30 adult I. dammini. Noninfected, female Dutch Belted O. cuniculus weighing less than 1 kg were used in experiments requiring a second cycle of transmission. To detect spirochetal infection in ticks, their gut contents were smeared on ringed slides, allowed to air dry, and fixed in acetone for 10 minutes. All preparations were screened by direct fluorescent microscopy (DFA) using a high-titred, fluorescein isothiocyanate conjugated polyclonal antibody to B. burgdorferi, using epifluorescent illumination at 400X as previously described (Burgdorfer et al, 1982). Slides prepared from Peromyscus-derived, infected nymphal I. dammini were included as positive controls. An indirect immunofluorescence (IFA) procedure was used to detect antibody to B. burgdorferi. Thus, antigen (BSK cultured spirochetes of the Guilford Strain, originally isolated in Connecticut by A.C. Steere) was immobilized in wells of IFA slides by allowing the slides to dry and then fixing in acetone. Test serum was diluted ten-fold from 1:10 to 1:10,000 and applied in duplicate to the wells. Slides were incubated at 37^{o}C for 30 min in a humid chamber. After washing in PBS, FITC anti-rabbit IgG (Sigma) was added to each well at a concentration of 1:100. IFA slides were again incubated, washed and then examined by epifluorescent microscopy. Sera from infected

as well as noninfected rabbits were included for comparison in each test.

B. Results

In a preliminary study, a diagnostic reagent commonly used to characterize B. burgdorferi was applied to the isolate of spirochetes used in these studies. Gut contents of adult I. dammini, derived from the collection of ticks that was used to establish our experimental isolate, were screened by dark-field microscopy until 5 infected ticks were found. Preparations from each were fixed and prepared for antigen detection by indirect immunofluorescence using a monoclonal antibody preparation (H5332, obtained from A.G. Barbour). Spirochetes in all preparations reacted intensely with the reagent. We then determined whether spirochetes were ingested by larvae of rabbit-feeding I. dentatus and whether they survived through the larval-nymphal molt; survival was compared to that in I. dammini. Following the standard time-course of infection reported for rabbits (Burgdorfer 1984), larvae of both species were applied in separate ear bags 10 days after all infecting adult ticks had fed to repletion. In each experiment, infection in derived nymphal I. dammini was matched to that in a corresponding cohort of I. dentatus. Although most of the I. dammini became infected, less that half of the I. dentatus did so (Table 5). Thus, Lyme disease spirochetes readily infect and survive in I. dentatus, but not as frequently as in I. dammini (X^2 $p < 0.05$, d.f.=5).

To determine whether I. dentatus may transmit spirochetal infection, one nymphal tick (from cohort number 5 listed in Table 5) that had fed as a larva on an experimentally infected rabbit was placed on each of 2 noninfected rabbits. The infecting ticks were each recovered after becoming replete, then dissected and examined for spirochetal infection; only one of these 2 ticks contained spirochetes in its gut (Table 6). To determine whether these rabbits had become infected by I. dentatus-borne B. burgdorferi, cohorts of larval I. dammini were placed on them 10 days after the infecting ticks became engorged. In an independent measure of serological conversion in these rabbits, blood was sampled aseptically from the central ear vein of each at the beginning of the experiment as well as after the "xenodiagnostic" larvae were applied. Serological evidence thereby confirmed the xenodiagnostic evidence of infection in this rabbit that had been bitten by an infected tick. Thus, I. dentatus is capable of transmitting spirochetal infection to rabbits.

C. Discussion

These experiments constitute the first proof of vector competence for B. burgdorferi in a tick other than one of

the I. ricinus complex of species. Although the subgenus Ixodes includes both I. dentatus and the various members of the I. ricinus complex, these ticks are morphologically quite distinct (Cooley and Kohls 194/). The spirochete has been isolated from other ticks, as well, including species of the genera Amblyomma (Schulze et al., 1985), Dermacentor (Anderson et al. 1986) and Rhipicephalus (Rawlings 1987) and a variety of insects (Anderson 1987). Experimental proof of vector competence in these other arthropods, however, has not been demonstrated.

We achieved successful transmission of B. burgdorferi in one of two trials, a rate that is consistent with the (64%) prevalence of infection in the nymphal ticks that were used. Although additional trials would be required to quantify vector competence, successful infection of a rabbit, using an experimentally infected tick, amply demonstrates that I. dentatus is capable of transmitting B. burgdorferi. The identity of the spirochete used in these experiments seems certain. The possibility of a naturally rabbit-infesting spirochete was excluded because the strain was isolated from an island on which rabbits rarely were seen. The I. dammini ticks that, themselves, inoculated our original isolate, maintained an intense natural zoonosis of Lyme disease there in mice (Piesman et al 198/). Indeed, these spirochetes reacted strongly with the immunological reagents that were applied, including a monoclonal antibody. Our experimental material fulfills the generally accepted criteria for B. burgdorferi. Because I. dentatus will sometimes parasitize birds, this tick can serve as a vehicle for transporting the agent of Lyme disease to new sites in eastern North America. In this region, subadult I. dammini, too, parasitizes birds. Transport would be facilitated, however, if both ticks were available as vehicles of transmission. Indeed, I. dentatus has been collected from diverse locations including Alabama, Oklahoma and West Virginia (Clifford and Keirans, 1974) in which isolated human infections occur and in which I. dammini is not endemic (Schmid, 1985). This disjunct distribution may reflect transport on birds.

Its extreme host-specificity provides I. dentatus with potential as an effective maintenance vector for the Lyme disease spirochete, even if it were less competent as a vector for mice than is I. dammini. Narrowness of host-range would contribute geometrically to vectorial capacity (Spielman & Rossignol 1984). Focal enzootic cycles of transmission of B. burgdorferi may be maintained by various Ixodes ticks. Such host-specific, mouse-feeding Ixodes as muris in eastern North America (Smith 1944), angustus in the West (Fay and Rausch 19//) or trianguliceps in western Europe (Hussain 197/), provide potential for transmission of silent infections that may become intensely zoonotic if a less host-specific member of the ricinus complex is present.

Indeed, the present I. dammini-borne zoonosis may have emerged when the recent proliferation of deer in North America permitted this species to replace I. muris (Spielman et al 1985). That potential would be more remote if the vector were to focus its feeding on hosts that infrequently are parasitized by I. ricinus-like ticks. Human infection correspondingly would be infrequent if I. cookei or I. texanus alone were to maintain transmission in sciurids. Unlike the mouse-feeding species, listed above, these ticks have been found attached to people (Clifford and Keirans, 1974). In eastern North America, rabbit-feeding Ixodes ticks support a cycle of B. burgdorferi that indirectly affects people by providing a reservoir of infection for mice.

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APPENDIX

Fig. 1 Borrelia burgdorferi (B) in the lumen of the gut of a nymph from group 1.25 weeks postlarval feeding, fed for 48 h on an uninfected hamster. The spirochetes are located in the endoperitrophic space of the gut mixing with the ingested blood consisting of intact (IER) and lysed (LER) erythrocytes. X 63 250.

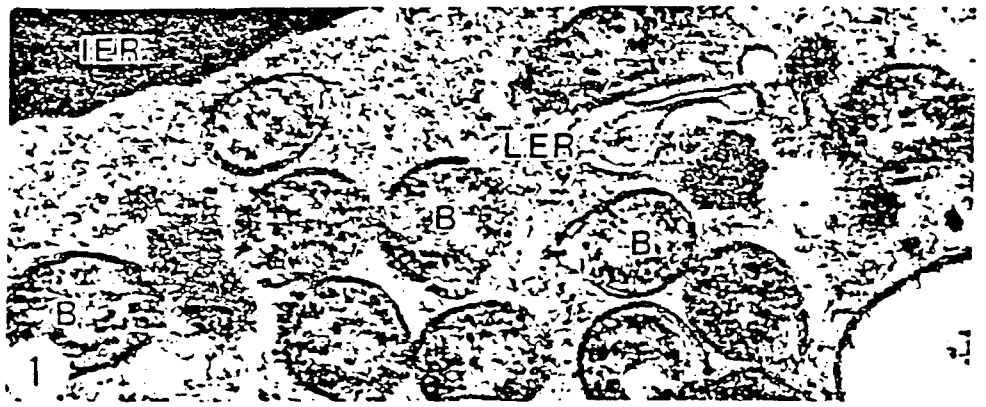

Fig. 2 Higher magnification of cross section of a spirochete. Tubular peri-plasmic flagella (f) are seen within the periplamsic space (ps) located between the outer envelope (oe) and the protoplasmic cylinder (c). Numerous ribosomes (r) and a clearer nuclear area (n) are seen within the protoplasmic cylinder. pm, plasma membrane; cw, cell wall. X 93 950.

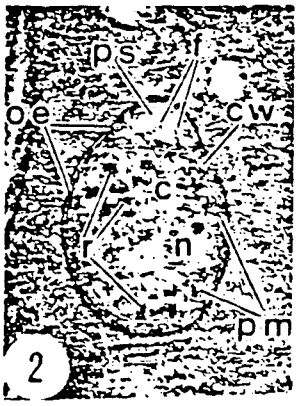

Fig. 3 Longitudinal section of a single spirochete (B) lying within the ectoperitrophic space (EC) in close contact with the microvilli (MV) of a gut epithelial cell (E). Note the helical shape of the spirochete and the periplasmic flagella (f) running along the length of the cell. Spirochetes in cross section are seen as well (arrows). PM, peritrophic membrane. X 56 370.

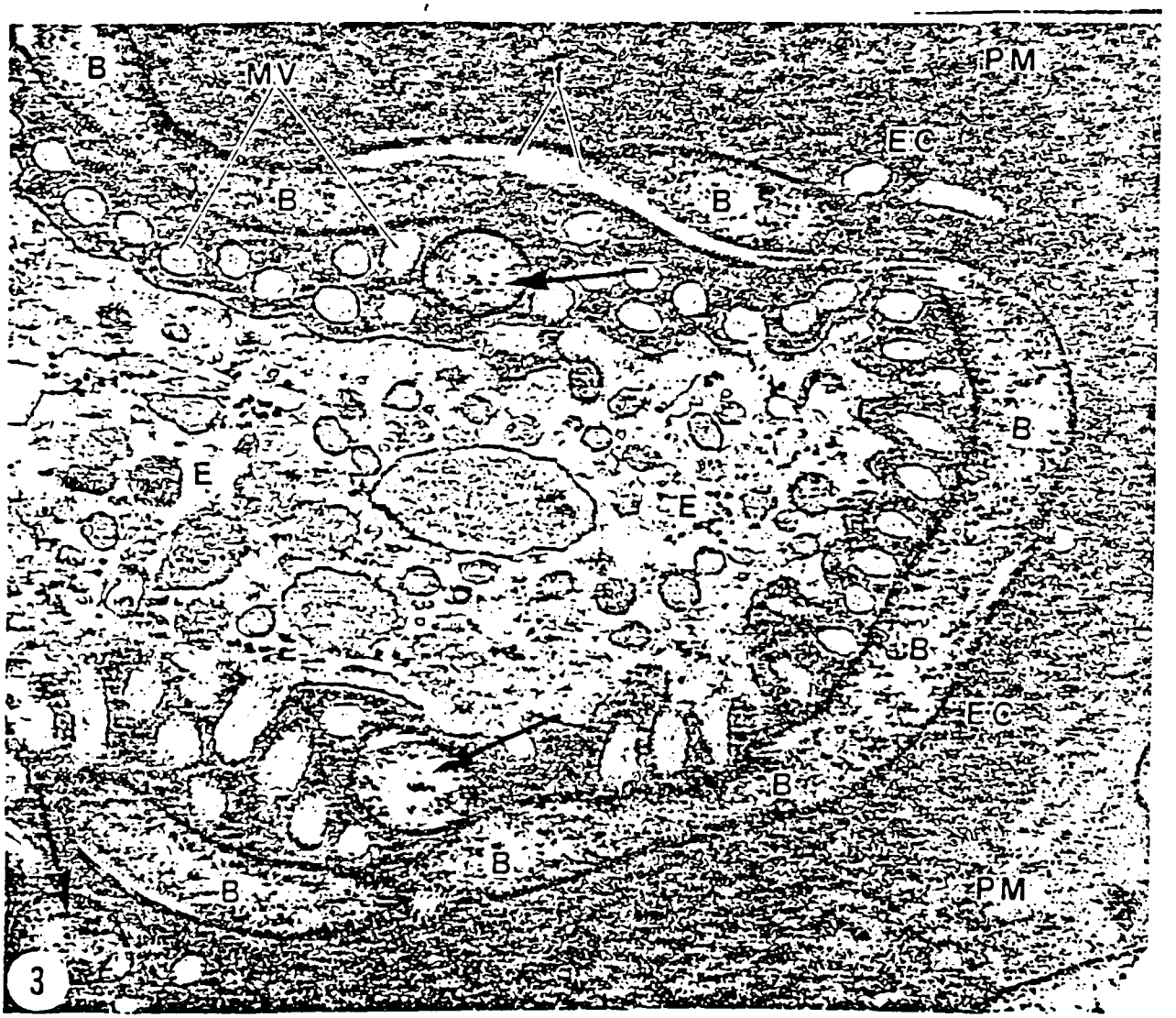

Fig. 4 Borrelia (B) penetrating the apical surface of the gut epithelium (E) between the microvilli (MV). L, lumen. X 46 975.

Fig. 5 Spirochete (B) lying within the apical gut epithelium (E). Note the added outer membrane (OM) presumably derived from the apical gut plasma membrane (P). MV, microvilli; L, lumen. X 75 900.

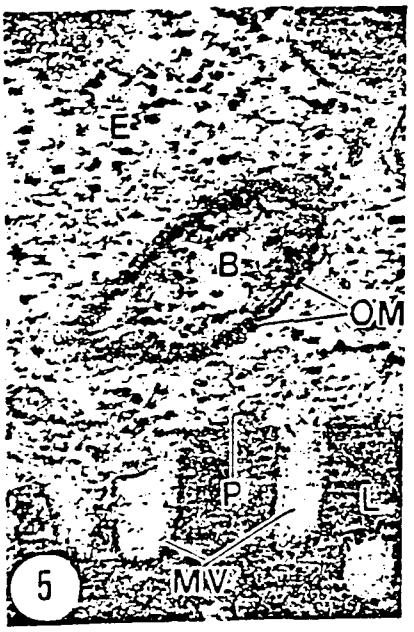

Fig. 6 After 12 h feeding on an uninfected hamster, the spirochete (B) is located intercellularly in the basal surface of the gut between two adjacent epithelial cells (E). The plasma membraned (arrows) are pushed apart by the spirochete. BL, basal lamina; M, external gut musculature; H, hemocoel. X 37 375.

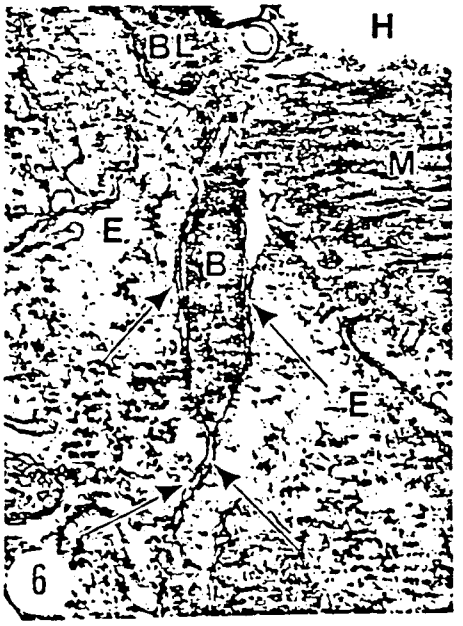

Fig. 7 Intercellular Borrelia (B) sandwiched between two epithelial cells (E) midway between the apical and basal surfaces of the gut epithelium. Note how the plasma membranes (arrows) of the adjacent epithelial cells are pushed apart by the spirochete. X 44 850.

Fig. 8 Borrelia (B) located extracellularly between the basal plasma membrane of the gut (arrows) and the basal lamina (BL); oe, outer envelope; H. hemocoel. X 44 850.

Fig. 9 Borrelia (B) crossing the basal lamina (BL) of the gut into the hemocoel (H). E, gut epithelial cell; L, lumen; MV, microvilli. X 44 850.

Fig. 10 Borrelia (B) with an outer amorphous coat (ac) in the homocoel (H) adhering to the basal lamina (BL) of a type I salivary acinus. X 46 975. Inset: Enlarged serial section of the spirochete showing the amorphous outer coat (ac). X 101 500.

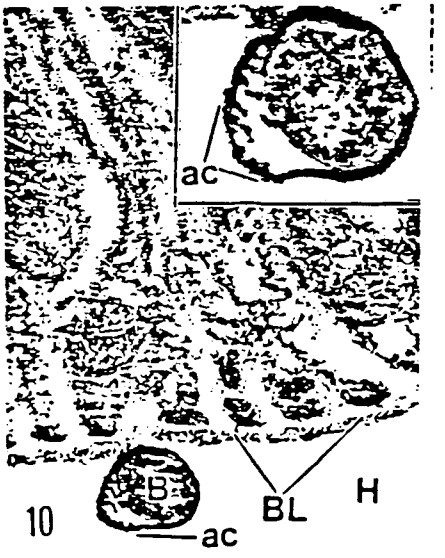

Fig. 11 The spirochete (B) within the large extracellular spaces (arrows) near the basal surface of a type I salivary acinus. BL, basal lamina; H. hemocoel. X 44 850

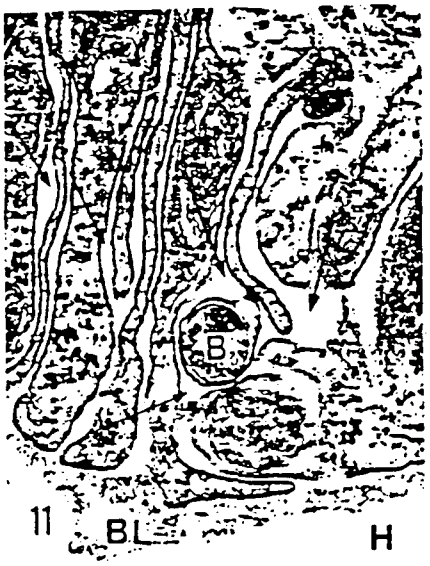

Fig. 12 Borrelia (arrowheads) located intercellularly near the apical surface of a glandular salivary acinus. Apical microvilli (MV) extend into the lumen (L) of the acinus. X7575.

Fig. 13 Borrelia (arrowheads) penetrating into the lumen (L) of a glandular salivary acinus. X 10 560.

Fig. 14 Borrelia (B) free within the lumen (l) of a glandular salivary acinus. MV, vicrovilli; ac, amorphous outer coat. X 50 600.

Fig. 15 Borrelia (arrowhead) lying within the tissue of a salivary duct (D). A second spirochete (arrow) lies within a ganglion (G) located adjacent to the salvary duct. N, nucleus of salivary duct cell; BLS and BLG. basal laminae of salivary duct and ganglion, respectively. X 15 750. Inset: Enlargement of spirochete near basal surface of duct cell. X 63 000.

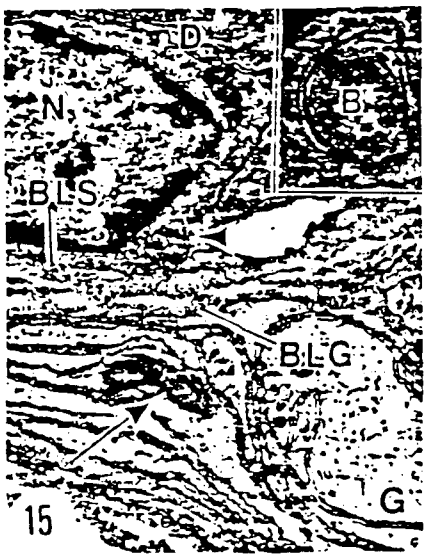

Fig. 16 Borrelia (arrow) located within the lumen (L) of a salivary duct. N, nucleus of duct cell; Cu, cuticle lining duct. X5300. Inset: Enlargement of spirochee outlined in lumen (L). X 26 500.

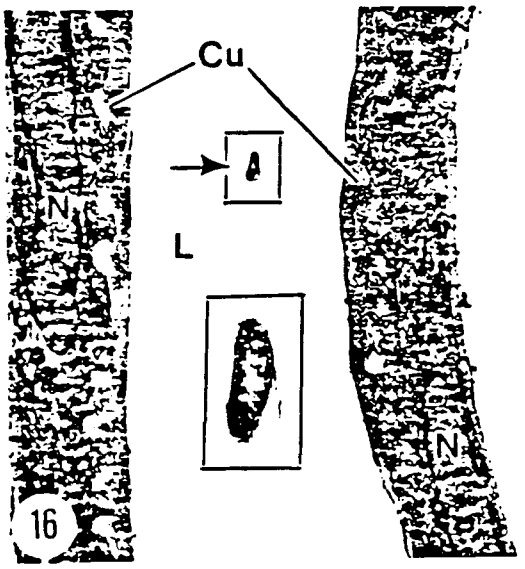

Fig. 17 Borrelia (B) in the hemocoel (H) in contact with the basal lamina (BL) of a nephrocyte (NE) . An outer amouphous coat (ac) can be seen covering the spirochetes. X 37 375.

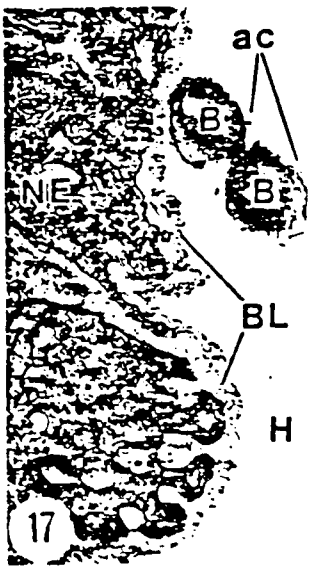

Fig. 18 Borrelia (B) in the brain tissue from a group 2 nymph, 37 weeks postlarval feeding, after 12 h feeding on an uninfected hamster. Numerous secretory vesicles (SV) are seen. X 29 450.

Fig. 19 Borrelia (B) in the lumen of the gut (L) in close contact with numerous microvilli (MV). E. gut epithelial cell; oe, outer envelope of spirochete. X 37 375.

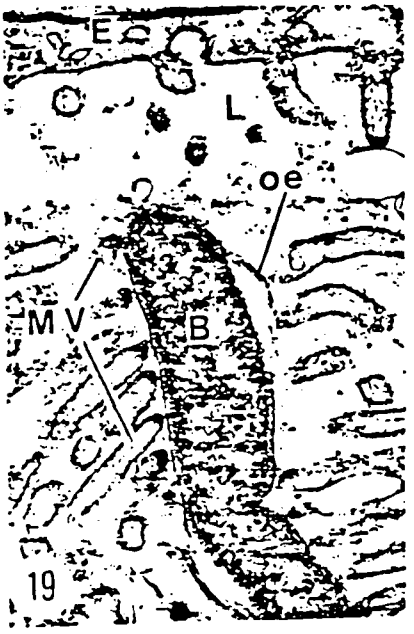

Fig. 20 Borrelia (B) located intercellularly near the basal surface of the gut. Adjacent plasma membranes (arrows) are pushed paprt to accommodate the spirochete. N. nucleus of the gut epithelial cell; BL, basal almina; M. external gut musculature; H, hemocoel. X 26 635.

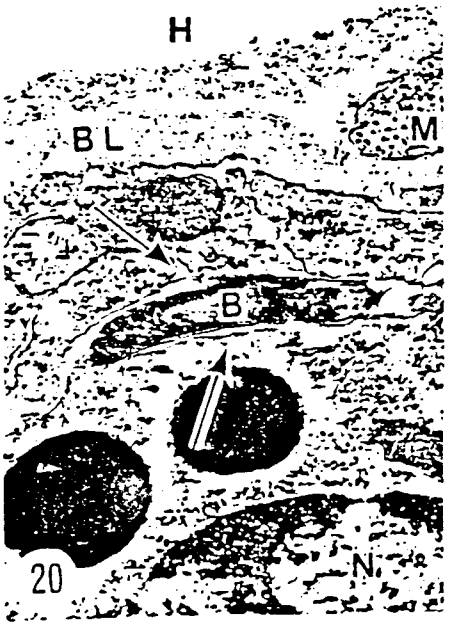

Fig. 21 Borrelia (arrows) located within the external gut musculature (M). H. hemocoel; BL, basal lamina of gut; E, gut epithelial cell. X 47 000.

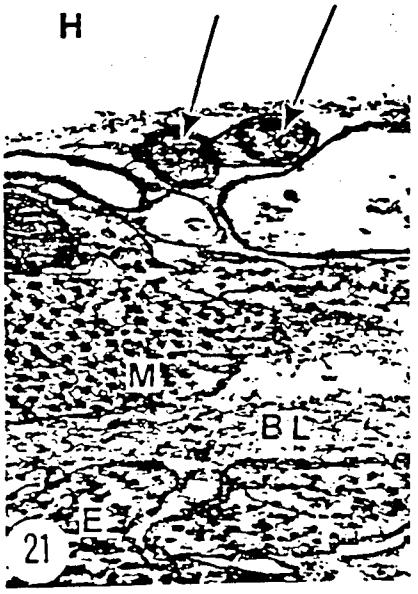

Fig. 22 Borrelia (arrows) within the basal surface of a glandular salivary acinus. BL, basal lamina of acinus; MI, mitochondria; SG, secretory granules.

Fig. 23 Unusual form of Borrelia seen in the gut lumen of an unfed numph form group 1, 35 weeks postlarval feeding. Five normal looking protoplasmic cylinders (c) are seen surrounded by a single outer envelope (oe). Flagella (f) are seen within the periplamsic space. A larger deteriorating from (arrowhead) is also seen. X 65 765.

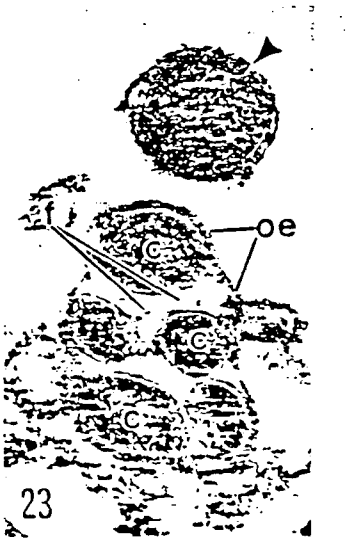

Fig. 24 Unusual form of Borrelia (B) seen near the basal surface of the gut in a group 2 nymph, 37 weeks postlarval feeding, fed for 12 h on an uninfected hamster. An intact outer envelope (oe) surrounds this large form which is more than five times the diameter of normal spirochetes seen in Fig. 21 at the same magnification. A second outer membrane (OM) surrounds the entire strucutre demonstrating the intracellular location of this giant form. The protoplasmic cylinder (c) seems to be deteriorating and fragments of flagella (f) are seen in the periplasmic space. X 47 000.

Fig. 25 Borellia (B1) is seen within the apical epithelium surrounded by a second outer membrane. Another spirochete (B2) is seen penetrating the apical gut epithelial surface from the ectoperitrophic space (EC). PM, peritophic membrane; MV, microcilli. X 29 450. Insets: (i) enlargement of spirochete (B1), arrowhead points to the second outer membrane surrounding the spirochete within the apical gut cytoplasm, X 50 000; (ii) enlargement of spirochete (B2), arrows point to the invaginating apical plasma membrane of gut epithelial cell (e), X 50 000.

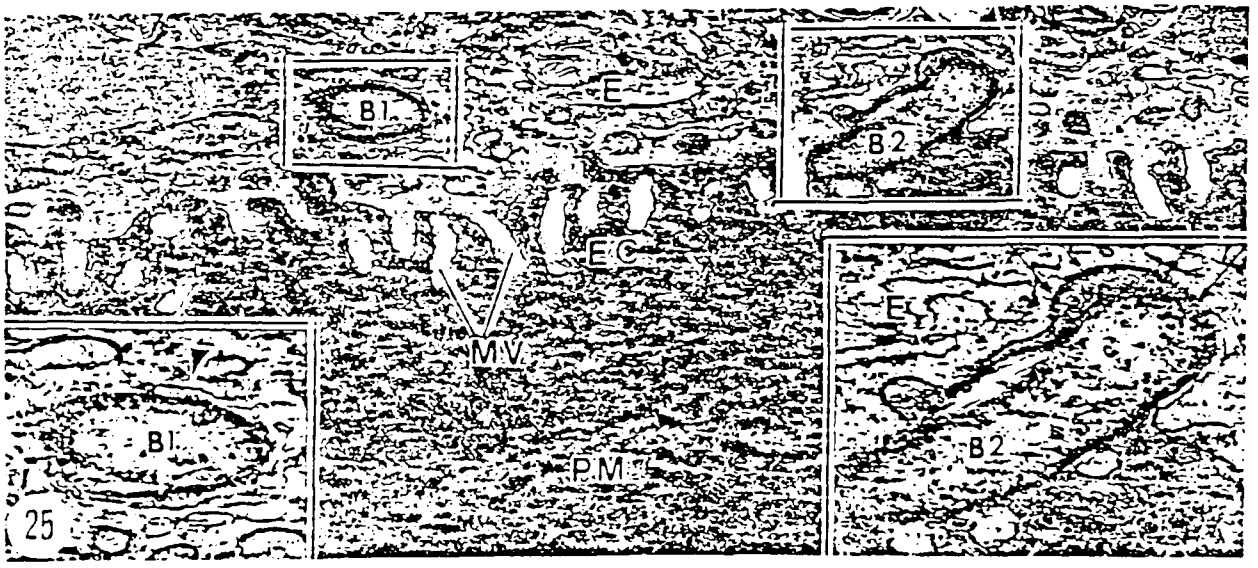

Fig 26 Numerous Borrelia (B) are seen in the lumen (L) of the gut. A peritrophic mebrane (PM) is clearly seen and several spirochetes (arrows) are seen crossing it. E, epithelial cell; EC, ectoperitrophic space. X 29 450.

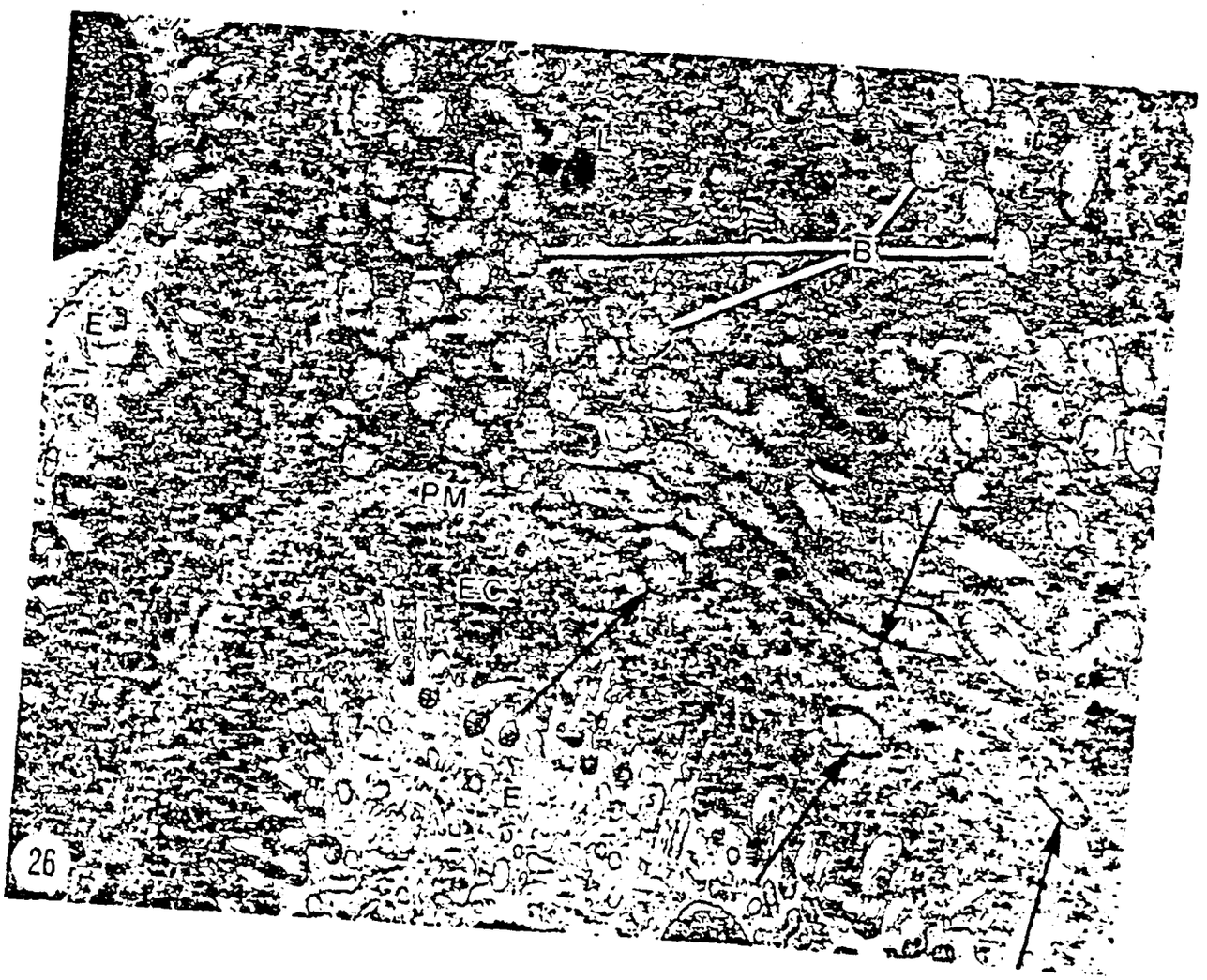

Table 1. Duration of time before Lyme disease spirochetes are ingested by attached ticks.

Days larvae attached to host	Kind of host	No. larvae tested	No. larvae infected	Mean spirochetes /larva	Mean spirochetes in skin
1	Peromyscus	5	0	0	-
		6	0	0	-
		6	0	0	-
		5	0	0	-
	Hamster	10	0	0	0
		8	0	0	0
2	Peromyscus	5	3	5.2	-
		5	0	0	-
		6	0	0	-
		5	0	0	-
	ICR mouse	5	1	7.0	-
		5	0	0	-
	Hamster	8	1	2.0	0
		9	2	8.5	0
3	Peromyscus	6	6	>100	-
		6	5	10.0	-
		6	2	1.5	-
		10	2	8.0	-
	ICR mouse	5	5	>100	-
		5	4	35.8	-
	Hamster	10	8	50.6	28
		10	9	40.2	18
Replete	Peromyscus	6	5	>100	
		6	6	>100	
		6	5	>100	
	ICR mouse	5	5	>100	
		5	4	>100	
	Hamster	6	6	78.3	
		6	5	57.4	

Table 2

Frequency of insemination of female Ixodes dammini collected from vegetation and from deer.

Collection			Spermatophore present		
Mode	Site	Month	No. deer	No. examined	%
Flagging	Ipswich	Apr	-	19	57.8
		Apr	-	30	63.3
		May	-	39	48.7
		May	-	18	72.2
		Oct	-	24	50.0
		Oct	-	26	46.0
"	Yarmouth	Oct	-	29	51.7
		Mar	-	19	31.5
		May	-	16	37.5
"	Nantucket	Apr	-	26	30.7
Deer	Ipswich	Nov	4	54	92.5
"	"	May	1	15	100
"	"	Nov	1	5	40.0
"	"	Nov	11	27	92.5

Table 3

Effect of insemination on degree of engorgement of female Ixodes dammini. Field-derived and laboratory reared ticks were variously exposed to males and permitted to engorge on the ears of rabbits. All engorged ticks produced eggs.

Source of fem.	Exposed to male	Number tot.	Number fed	Mean final weight mg.	Mean final weight SD	Frequency distribution of hatching per egg clutch 0%	25%	75%	100%
Reared	none	15	0	33.8	15.6	-	-	-	-
	pre-host on host	23	74	147.7	36.2	0	0	29	71
		21	81	298.1	99.1	0	0	24	76
field	none	25	52	78.7	116.8	0	0	23	77
	on host	25	92	197.3	88.7	0	0	13	87

Table 4

Sperm precedence in doubly inseminated female Ixodes dammini. Virgin ticks or ticks that had been confined with irradiated or with non-irradiated males were variously confined with such males while feeding on rabbits.

Condition of inseminating male			Mean weight engorged		Frequency distribution of hatching per clutch				
first	last	No.	mg	S.D.	0%	-25%	-50%	-75%	-100%
none	normal	20	117.2	82.1	0	0	0	3	17
none	irrad.	22	159.8	97.4	22	0	0	0	0
irrad.	normal	27	172.6	105.1	4	2	0	3	18
normal	irrad.	29	214.4	110.2	21	4	0	0	4
none	both	39	282.1	63.9	19	3	17	0	0

Table 5

Infectivity to ticks of rabbits infected by Lyme disease spirochetes, and a comparison of the vector competence of subadult I. dentatus and I. dammini. Adult I. dammini, collected where Lyme disease was prevalent, were used to infect laboratory rabbits which later served as host to larval I. dammini and I. dentatus. Evidence of infection was sought in the resulting nymphs.

	Spirochetes in nymphal Ixodes			
	dentatus		dammini	
Rabbit number	No. observed	% infected	No. observed	% infected
1	5	60	5	80
2	5	0	5	0
3	4	50	6	83
4	4	25	4	100
5	11	64	5	80
6	6	67	4	100
7	10	40	5	60
Total	45	47	34	71

Table 6

Infectivity to rabbits of I. dentatus infected by Lyme disease spirochetes. One nymphal I. dentatus, developing from a larva that engorged on a laboratory rabbit infected by Lyme disease spirochetes, was used to infect individual laboratory rabbits which later served as host to larval I. dammini. Evidence of infection was sought in the resulting nymphs and by serological criteria.

Spirochetes in tick used to infect rabbit	Spirochetes in derived nymphs		Seropositive at 1:100	
	No.	% infected	before bite	after bite
yes	8	12	no	yes
no	12	0	no	no

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